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A Novel Serotype-Specific Gene Cassette (*gltA-gltB*) Is Required for Expression of Teichoic Acid-Associated Surface Antigens in *Listeria monocytogenes* of Serotype 4b

XIANG-HE LEI,[1]† FRANZ FIEDLER,[2] ZHENG LAN,[1] AND SOPHIA KATHARIOU[1]*

Department of Microbiology, University of Hawaii, Honolulu, Hawaii 96822,[1] and Institute for Genetics and Microbiology, University of Munich, Munich, Germany[2]

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***Listeria monocytogenes* serotype 4b strains account for about 40% of sporadic cases and many epidemics of listeriosis. Mutations in a chromosomal locus resulted in loss of reactivity with all three monoclonal antibodies (MAbs) which were specific to serotype 4b and the closely related serotypes 4d and 4e. Here we show that this locus contains a serotype 4b-4d-4e-specific gene cassette (3,071 bp) which consists of two genes, *gltA* and *gltB*, and is flanked by palindromic sequences (51 and 44 nucleotides). Complete loss of reactivity with the three serotype-specific MAbs resulted from insertional inactivation of either *gltA* or *gltB*. The *gltA* and *gltB* mutants were characterized by loss and severe reduction, respectively, of glucose in the teichoic acid, whereas galactose, the other serotype-specific sugar substituent in the teichoic acid, was not affected. Within *L. monocytogenes*, only strains of serotypes 4b, 4d, and 4e harbored the *gltA-gltB* cassette, whereas coding sequences on either side of the cassette were conserved among all serotypes. Comparative genomic analysis of a serotype 1/2b strain showed that the 3,071-bp *gltA-gltB* cassette was replaced by a much shorter (528-bp) and unrelated region, flanked by inverted repeats similar to their counterparts in serotype 4b. These findings indicate that in the evolution of different serotypes of *L. monocytogenes*, this site in the genome has become occupied by serotype-specific sequences which, in the case of serotype 4b, are essential for expression of serotype-specific surface antigens and presence of glucose substituents in the teichoic acids in the cell wall.**

Numerous serotypes of *Listeria monocytogenes* have been identified using the antigenic scheme of Seeliger and Hoehne (16). However, three serotypes, 1/2a, 1/2b, and 4b, account for more than 95% of clinical isolates (5). Serotype 4b is of special interest, as it is implicated in about 40% of sporadic cases and the majority of epidemics of food-borne listeriosis reported in Europe and North America during the past 20 years (1, 7, 15). This may reflect relatively high virulence of serotype 4b strains for humans, although unique pathogenesis attributes of this serotype have not yet been identified.

The somatic component of the serotypic designation in *Listeria* resides primarily in the anionic polymer, teichoic acid (TA), which consists of polyribitol phosphate and is covalently linked to peptidoglycan (4, 6, 18). Glycosidic substitution(s) of the ribitol phosphate units render the TA variable, structurally and antigenically, among different serotypes. In serogroup 1/2 (e.g., serotypes 1/2a and 1/2b), *N*-acetylglucosamine and rhamnose are present as substituents on the ribitol, whereas in serogroup 4, *N*-acetylglucosamine is integral to the TA chains. A unique glycosidic substitution pattern is present in serotype 4b, where the integral *N*-acetylglucosamine bears both galactose and glucose substituents (4, 18).

In an effort to develop tools useful for the identification of antigenic and genetic attributes unique to serotype 4b bacteria, we have used monoclonal antibodies (MAbs) (c74.22, c74.33, and c74.180) which reacted with strains of serotypes 4b, 4d, and 4e (referred to collectively as serotype 4b-4d-4e) (8) to identify serotype-specific genomic regions. One such region was shown to harbor the serogroup 4-specific gene *gtcA*, which has been recently described (14). Insertional inactivation of *gtcA* resulted in loss of reactivity with one of the MAbs (c74.22), loss of galactose, and marked reductions in the glucose in the TA of the cell (14). A different genomic region was found to be specific to serotypes 4b, 4d, and 4e, and mutants in this region lacked reactivity with all three MAbs (10). Here we report the cloning and characterization of the genes composing this region and provide genetic evidence for their involvement in serotype-specific surface antigen expression and TA glycosylation in *L. monocytogenes* serotype 4b.

MATERIALS AND METHODS

Bacterial strains and media. *Listeria* and *Escherichia coli* strains were grown and preserved as described before (14). Antibiotics used for *Listeria* and for *E. coli* were as described before (14). Generation of transposon mutants of the serotype 4b strain 4b1 and screening of the mutants with the MAbs have been described elsewhere (10).

Biochemical analysis of cell wall composition. Cell wall composition was determined as described by Fiedler et al. (4). TA from *Listeria* was prepared and analyzed as previously described (4, 6).

Molecular procedures. Procedures for extraction of plasmid DNA from *E. coli* and genomic DNA from *Listeria* and for nonradioactive labeling and detection of DNA were previously described (10). Fragment XL7-1, which flanks the single transposon insertion in mutant XL7, has been described elsewhere (10). This fragment was sequenced, and inverse PCR (13) was employed to obtain genomic fragments on either side, using as template genomic DNA of the wild-type strain 4b1 digested with *Eco*RI or *Sau*3A, purified from low-melting-point agarose with phenol-chloroform extractions (2), and self-ligated. Amplified fragments were cloned in pCR2.1 (Invitrogen) and sequenced. Sequence information was used to design new primers at the end of the known sequence for additional inverse

* Corresponding author. Present address: Department of Food Science, Food Pathogens Laboratory, North Carolina State University, Raleigh, NC 27695. Phone: (919) 513-2075. Fax: (919) 515-7124. E-mail: skathar@unity.ncsu.edu.

† Present address: Biolog, Hayward, CA 94545.

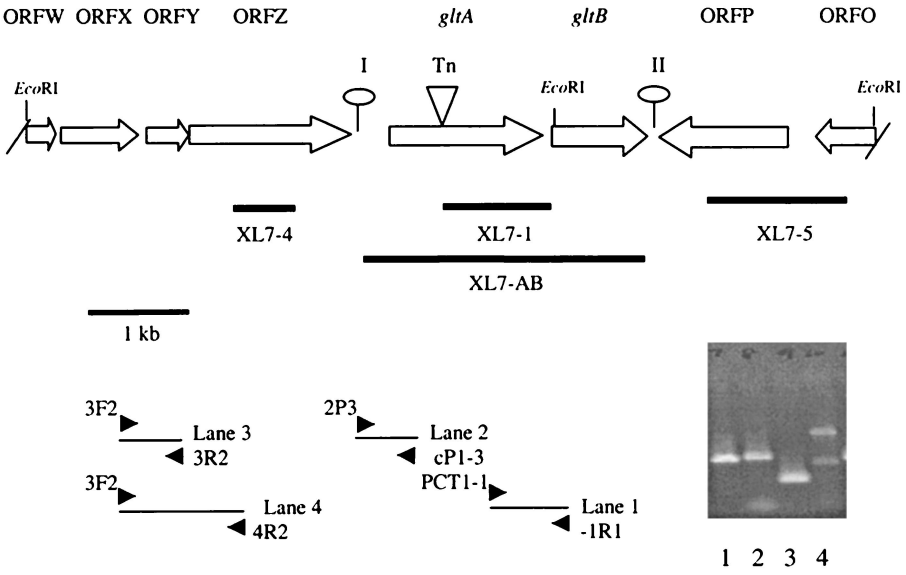

FIG. 1. Genomic organization of the region harboring the transposon insertion in mutant XL7. Open arrows indicate ORFs and predicted direction of transcription. Slashes at the borders indicate that ORFW and ORFO are partial; lollipops represent putative stem-loop structures. The location of the Tn*916*ΔE insertion in *gltA* (Tn) is indicated by a triangle. Thick lines represent DNA fragments used as probes in Southern blots, and arrowheads at the bottom indicate primers used in RT-PCR. RT-PCR was done as described in Materials and Methods with -1R1 as the primer for cDNA synthesis. The gel shows products of PCRs with cDNA as the template and the primer pairs -1R1–PCT1-1 (lane 1), cP1-3–2P3 (lane 2), 3R2-3F2 (lane 3), and 4R2-3F2 (lane 4). Negative controls (using RNA instead of cDNA as the template and the same pairs of primers) were devoid of any product (data not shown).

PCRs. Transposon-flanking fragments from other mutants were amplified using the Tn*916* terminal primer OTL (5′-CGG AAT TCC GTG AAG TAT CTT CCT ACA G-3′) with a 5′-end *Eco*RI site (underlined) and primer cP1 (5′-CAC AGA AGC GAT ACG ATG A-3′).

Probe construction. Probe locations are shown in Fig. 1. Probe XL7-1 (1.1 kb), which flanks the transposon insertion in mutant XL7, has been described elsewhere (10). Probe XL7-4 (0.6 kb) is internal to open reading frame Z (ORFZ) and consists of a 0.6-kb *Sau*3A fragment cloned into pUC19. Probe XL7-5 (1.6 kb), which includes ORFP and part of ORFO, was obtained as a PCR fragment with primers −1F5 (5′ CCG ACT GTA TCT TCT TTT CC 3′) and −1R9 (5′ TTT GCT ACT CAA CGG AGC CAC 3′) and 4b1 DNA as the template. The XL7-AB probe (2.9 kb), which includes both *gltA* and *gltB*, was obtained as a PCR fragment using primers 2P3 (5′-GTA ACG TCT CAT ATA GGG AG-3′) and −1R5 (5′-GTA GAA CAA TTG TAG TAC CG-3′). DNA fragments were isolated from low-melting-point agarose gels, purified by phenol-chloroform extractions (2), and labeled with a Genius kit.

RT-PCR. Procedures for RNA extraction from *Listeria*, construction of cDNA, and reverse transcription PCR (RT-PCR) were as described elsewhere (14).

Construction of integration mutant in *gltB*. To construct an integration mutant in *gltB*, an internal fragment of the gene was cloned in the temperature-sensitive shuttle vector pKSV7 (17), and integrants were selected by growth at the restrictive temperature (43°C) in medium containing chloramphenicol (CM medium) as follows. The internal fragment was amplified with primers −1F2 (5′-TTG GTA ACT CAC TAG TAC GT) and −1R4 (5′-ACA AGC ACA AAC AAA GAC GC), cloned in pCR2.1, recovered by *Eco*RI digestion, and subcloned into *Eco*RI-digested and dephosphorylated pKSV7. The resulting recombinant was electroporated into electrocompetent cells of the parental strain 4b1 as previously described (14), and transformants were isolated on CM medium after 48 h at 30°C. Integrants were isolated following four consecutive passages in CM medium at the restrictive temperature (43°C) and confirmed by Southern blotting. For colony immunoblots, the cultures were grown at room temperature.

Construction of pKA and pKAB. *Listeria* DNA fragments harboring *gltA* and *gltA-gltB* were amplified from DNA of the parental strain 4b1 by PCR using High Fidelity enzyme (Roche). Fragment A (containing *gltA*) was obtained by PCR using primers 2P3 and −1R1 (5′-CAA GGC AAG AGT ACA GCT AC-3′). Fragment AB (containing *gltA-gltB*) was amplified using primers 2P3 and −1R5 (described above for construction of the *gltA-gltB* probe XL7-AB), which had a *Hin*dIII site and a *Bam*HI site, respectively, at the 5′ end. The PCR fragments were excised from low-melting-point agarose gels, purified with phenol-chloroform, and cloned into pCR2.1. Fragment A was isolated following digestion of the recombinant plasmid with *Eco*RI and was subcloned into pKSV7 which had been digested by *Eco*RI and dephosphorylated. Fragment AB was obtained following digestion of the plasmid with *Bam*HI and *Hin*dIII and directionally cloned into pKSV7 digested with the same enzymes. The resulting plasmids, consisting of pKSV7 with inserts of *gltA* and *gltA-gltB*, were named pKA and pKAB, respectively. Upon electroporation, 100 μl of the cells was plated on CM medium, and the plates were incubated at 30°C for 3 to 4 days.

Cloning of serotype 1/2b sequences. Primers 2P2 (5′- GAC CAT ATC GTC GTG CTA CA-3′) and −1R65 (5′-CGA GCA TAC AAG TGC TCG TT-3′) were used to amplify a 1.1-kb DNA fragment with DNA of strain F4242 (serotype 1/2b) as the template. The 1.1-kb PCR product was directly cloned into pCR2.1 and sequenced on both strands.

DNA sequencing and sequence analysis. Nested deletions were generated using the Erase-a-Base system (Promega) as suggested by the vendor. DNA was sequenced and analyzed as previously described (14).

Nucleotide sequence accession number. The nucleotide sequence data for *L. monocytogenes* serotypes 4b and 1/2b have been deposited in GenBank under accession numbers AF033015 and AF033016, respectively.

RESULTS

Mutants negative for serotype-specific MAbs. The single-insertion Tn*916*ΔE mutant XL7 lacked reactivity with all three serotype-specific MAbs (C74.22, C74.33, and C74.180) but had no readily detectable phenotypic differences from its wild-type counterparts in terms of growth at 20 and 35°C, motility, sensitivity to serotype-specific phage 2671 or *Listeria*-specific phage A511, hemolytic activity, and colony or cellular morphology. Furthermore, four additional independent transposon mutants (33N1, 33N2, 33N3, and 8A3) phenotypically identical to XL7 were found to harbor transposon insertions in the same *Eco*RI and *Hin*dIII genomic fragment as XL7 (10), suggesting that the MAb-negative phenotype of XL7 was associated with the Tn*916*ΔE insertion. DNA sequence analysis of XL7-1 and of the additional fragments derived by inverse PCR showed that the transposon was inserted in an ORF

termed *gltA* (for glucose in teichoic acid). The transposon insertion sites in mutants 33N1, 33N2, 33N3, and 8A3 were within a 10-nucleotide (nt) region in *gltA*, which also harbored the insertion in XL7. The target sequence for the transposon insertions conformed to the consensus target sequence (T[T/A]TTTTNNNNNNAAAAA[A/T]A) for Tn*916* (11).

Genomic organization and ORF analysis of the *gltA-gltB* region. Sequence analysis revealed six complete ORFs (ORFX, ORFY, ORFZ, *gltA, gltB*, and ORFP) and two partial ORFs (ORFW and ORFO) in this region (Fig. 1). ORFW (partial), ORFX, ORFY, ORFZ, *gltA*, and *gltB* were transcribed in the same direction and convergently to ORFP and ORFO (partial). Two palindromic sequences with the potential to form pronounced stem-loop structures flanked the *gltA-gltB* region. The palindrome for putative stem-loop I (51 nt; calculated free energy of formation, −46 kcal/mol) was in the region between ORFZ and *gltA*, 55 nt downstream of ORFZ and 279 nt upstream of *gltA*, whereas that for putative stem-loop II (44 nt; calculated free energy of formation also −46 kcal/mol) was 8 and 27 nt downstream of *gltB* and ORFP, respectively (Fig. 1). The organization of the region suggests that stem-loops I and II may serve as transcription terminators for ORFZ and ORFP, respectively.

The G+C contents of *gltA* and *gltB* were 34 and 34.8%, respectively, lower than is typical for *L. monocytogenes* (38%). In contrast, the other ORFs in this region had G+C contents noticeably higher than those of *gltA* and *gltB*: ORFW, ORFX, ORFY, and ORFZ had G+C contents of 38.8, 39.7, 41.5, and 39.8%, respectively, whereas the values for ORFP and ORFO were 39.6 and 40.6%, respectively.

***gltA-gltB* region.** The transposon-harboring ORF (*gltA*) (1,647 bp) was 386 nt downstream of ORFZ. We were unable to identify sequences upstream of *gltA* with detectable similarity to the canonical Shine-Dalgarno ribosome recognition sequences. A putative −10 promoter element (TATTAT) was identified 92 nt upstream of the putative start codon of *gltA*. The coding sequence of *gltA* appears to be novel, as screens of the nucleotide and protein databases failed to identify sequences with significant homology to either the gene or the deduced gene product. The latter (548 amino acids, calculated M_r of 62,755, pI 9.0) may be membrane associated in *L. monocytogenes*, as hydrophobicity analysis of the deduced polypeptide revealed 11 putative transmembrane segments (data not shown).

Immediately downstream of *gltA* was *gltB* (948 nt). The *gltA-gltB* intergenic space was only 10 nt, and the putative Shine-Dalgarno site preceding *gltB* (AGGAGAGA) included the last nucleotide of the *gltA* ochre codon, suggesting that the two ORFs may be translationally coupled. *gltB* had 57% identity over its entire length with *rfbj* (ORF10X5) and ORF10X9, which are adjacent to each other on the genome of *Shigella flexneri* (accession no. X71970). In *S. flexneri* this region has been shown to be involved in polymerization of lipopolysaccharide (12), although the exact functions of these two ORFs are unknown.

The deduced *gltB* gene product (315 amino acids, calculated M_r of 36,223, pI 6.04) contained two putative transmembrane domains (underlined in Fig. 2). Protein database searches showed significant similarity between the putative GltB and the deduced products of the *S. flexneri rfbj* and ORF10X9 (56 and 42% identity, respectively). The putative GltB also had 48% identity over its entire length with RfbJ of *Synechocystis* sp. strain PCC6803 (accession no. S77381) and lower (25 to 35%) identity with numerous glycosyltransferases and dolichol phosphate mannosyltransferases from bacteria and archaea. Figure 2 shows alignment of the deduced *gltB* gene product sequence with selected sequences.

Coding sequences upstream of *gltA-gltB* (ORFW to ORFZ). BLAST and motif search analysis of the deduced amino acid sequences of ORFW (partial), ORFX, ORFY, and ORFZ suggested that all had characteristics of ABC (ATP-binding cassette) transporters (3). A putative ATP/GTP-binding site motif A (P loop) was identified in the deduced sequences of ORFX (residues 217 to 225) and ORFZ (residues 368 to 375). The ORFW-ORFX and ORFX-ORFY intergenic spaces were 2 and 21 nt, respectively, whereas the stop codon of ORFY overlapped by one nucleotide with the putative start codon of ORFZ, suggesting that ORFY and ORFZ are translationally coupled.

Coding sequences downstream of *gltA-gltB* (ORFP and ORFO). FASTA and BLAST analysis of ORFP, located downstream of *gltB* and transcribed convergently, suggested that the deduced product may be a penicillin-binding protein (PBP), having 34 to 55% identity over the entire amino acid sequence with PBPs from numerous other bacteria. Highest similarity (55% identity) was observed with the D-alanyl-D-alanine carboxypeptidase, PBP5, of *Bacillus subtilis* (accession no. P08750). ORFP was preceded by ORFO (partial), transcribed in the same orientation as ORFP and separated from it by 214 nt. The deduced ORFO product had 49 and 46% identity over its entire available length (189 amino acids) with the 7-β-(4-carboxybutanamido)cephalosporanic acid acylase (glutaryl 7-amino cephalosporanic acid [7-ACA] acylase precursor) of *Bacillus laterosporus* and with the cocaine esterase of *Rhodococcus* sp. strain MB1, respectively. These similarities are difficult to evaluate at this time, as such enzymatic activities have not been detected before in *L. monocytogenes*.

Transcriptional studies. The quantitative levels of *gltA-gltB* transcripts were too low for reliable detection and size determination by Northern blotting (data not shown), and RT-PCR was used for transcriptional studies. When primer −1R1 (located in *gltB*) was used for reverse transcription, a PCR product of the expected size was obtained using primers −1R1 and PCT1-1 (spanning *gltB* and *gltA*) (Fig. 1, lane 1), suggesting that *gltA* and *gltB* were cotranscribed. Furthermore, the transcript contained the 386-nt region between *gltA* and ORFZ, which includes the palindromic sequence, as suggested by a product of the expected size with primers cP1-3 and 2P3 (located in the region between ORFZ and *gltA*) (Fig. 1, lane 2). Surprisingly, cDNA produced by primer −1R1 could be amplified by primer 3R2 (located in the 3′ region of ORFX) and either 3R2 or 4R2 (Fig. 1, lanes 3 and 4), suggesting that ORFY and ORFZ were included in the transcript as well. These and additional RT-PCR data (not shown) suggest the presence of transcripts harboring not only *gltA-gltB* but also extending through the relatively long (386-nt) region between *gltA* and ORFZ, to at least 2,287 nt upstream of *gltA*. *gltB* appears to be the last ORF in this transcriptional unit.

Insertional inactivation of either *gltA* or *gltB* results in absence of glucose in the TA, whereas galactose is not affected.

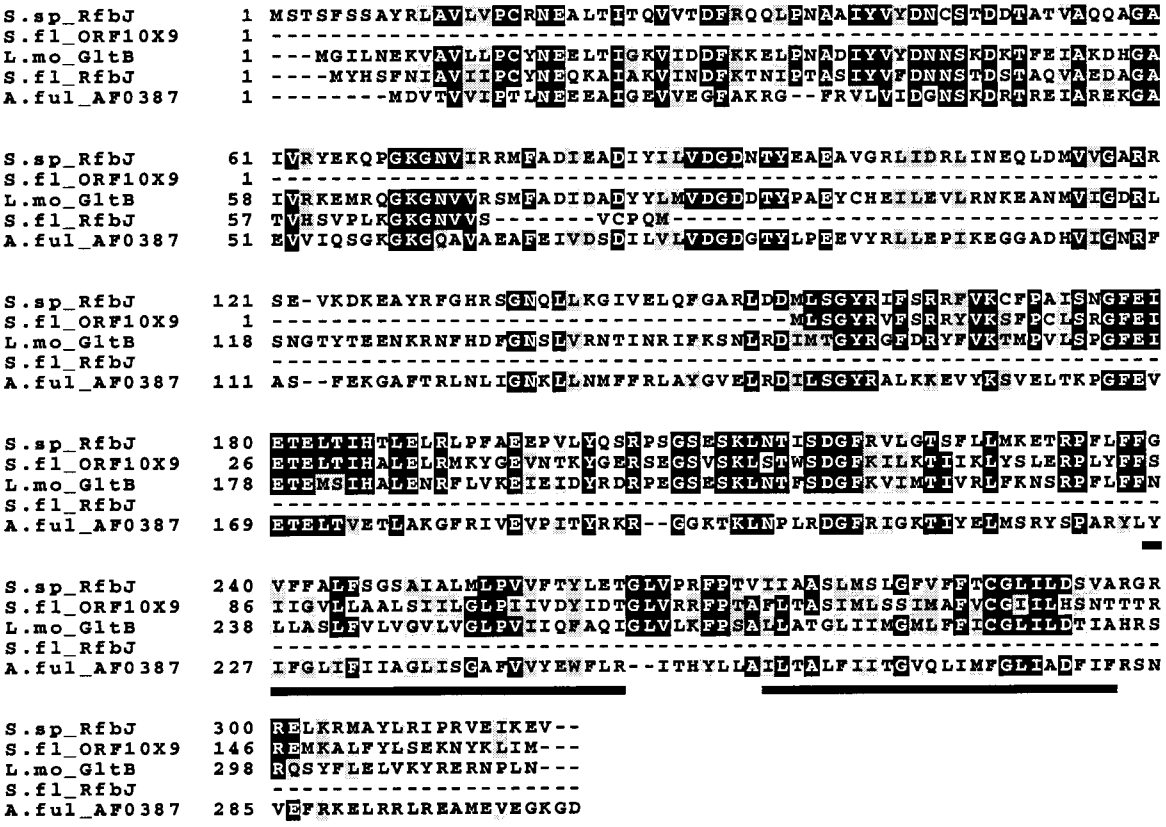

FIG. 2. Multiple sequence alignment (CLUSTAL) of the deduced sequences of, from top to bottom, RfbJ of *Synechocystis* sp. strain PCC6803 (accession no. S77381), ORF10X9 of *S. flexneri* (accession no. P37787), GltB of *L. monocytogenes* (accession no. AF033015), RfbJ of *S. flexneri* (accession no. P37786), and a putative glycosyltransferase of *Archaeoglobus fulgidus* (accession no. AE001078). The underlined segments represent predicted transmembrane regions.

Transposon mutants in *gltB* were not identified, and an integration mutant (4b1-INTB) was constructed, using the temperature-sensitive plasmid pKSV7. Similarly to XL7, the mutant had normal growth and other phenotypic characteristics but lacked reactivity with all three MAbs (data not shown). Biochemical analysis of TA from XL7 and 4b1-INTB showed that both mutants were severely deficient in glucose. In contrast to the wild-type parental strain 4b1, which had both galactose and glucose as substituents on the *N*-acetylglucosamine of the TA, as is typical of serotype 4b (4), glucose was undetectable in the TA of XL7 and present in only trace amounts in the TA of 4b1-INTB (Fig. 3). Interestingly, the other serotype-specific substituent, galactose, was present in normal amounts in the TA of the mutants, as were the integral components of TA (ribitol phosphate and *N*-acetylglucosamine) (Fig. 3). The loss of glucose in the TA of XL7 was also seen with the independently obtained *gltA* mutants 33N1, 33N2, 33N3, and 8A3 (data not shown).

The MAb-negative phenotype of XL7 can be partially complemented by *gltA* alone or in combination with *gltB*. The recombinant plasmids pKA and pKAB, harboring *gltA* alone and together with *gltB*, respectively, were electroporated into mutant XL7. Both plasmids included 219 nt upstream of the start codon of *gltA*, since a promoter may be contained within this region. The resulting strains were grown in the presence of chloramphenicol at 30°C, a temperature which permits both replication of the temperature-sensitive plasmid (17) and optimal expression of the serotype-specific surface antigens (8). Reactivity of the mutant with c74.22, c74.33, and c74.180 was restored partially and to the same levels by both plasmids, whereas XL7 harboring the shuttle vector pKSV7 alone remained negative with the MAbs (data not shown). Although pKA and pKAB partially restored reactivity with the MAbs, glucose in the TA of the mutant was not restored to detectable levels (data not shown).

***gltB* is needed for heterologous expression of the serotype-specific surface antigens in strains of serotypes 4a and 4c.** Strains of serotypes 4a and 4c lacked reactivity with the *gltA*-derived probe XL7-1 (10). When transformed with pKAB, strains ATCC 19114 (serotype 4a) and ATCC 19116 (serotype 4c) were rendered reactive with at least two of the MAbs, c74.22 and c74.33 (data not shown). When transformed by pKA these strains remained MAb negative, suggesting that *gltB* was required for expression of c74.22- and c74.33-specific surface antigens in these heterologous hosts.

Within *L. monocytogenes*, only strains of serotype 4b-4d-4e harbored sequences with homology to *gltA* and *gltB*, whereas ORFP and ORFZ were conserved among different serotypes. Hybridizations using probe XL7-AB, which contains both *gltA* and *gltB*, showed that the genes were unique to *L. monocytogenes* serotype 4b-4d-4e and could not be detected in DNA from strains of other serotypes. The genes have also been

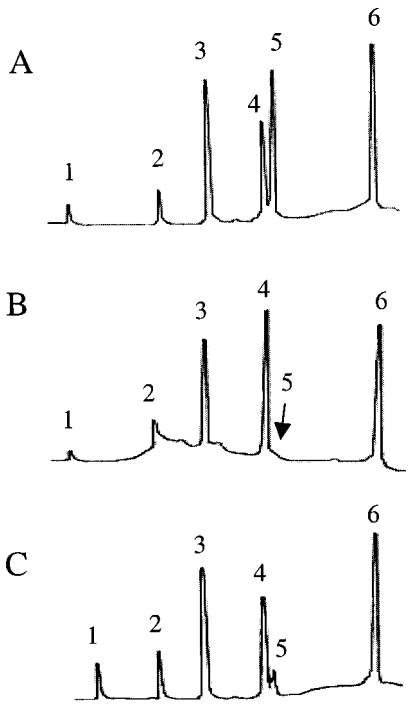

FIG. 3. TA composition of wild-type strain 4b1 (A), *gltA* mutant XL7 (B), and *gltB* mutant 4b1-INTB (C). TA preparation and analysis were done as described previously (4, 6). Peaks: 1, glycerol; 2, anhydroribitol; 3, ribitol; 4, galactose; 5, glucose; 6, glucosamine. The arrow indicates the position of the missing glucose peak in panel B.

detected in a unique lineage (lineage I) of *L. innocua* (9). *Eco*RI restriction fragment length polymorphisms using XL7-AB as the probe could differentiate between *L. monocytogenes* serotype 4b-4d-4e and *L. innocua* lineage I (Table 1). Southern blot and PCR data suggest that the *gltA-gltB* cassette was flanked by ORFZ and ORFP in *L. innocua* lineage I, as in *L. monocytogenes* serotype 4b (data not shown). No hybridization was observed with DNA from other *L. innocua* strains or other *Listeria* species (Table 1).

Southern blots using probes derived from ORFP-ORFO and ORFZ hybridized with all screened serotypes of *L. monocytogenes* suggesting that, in contrast to *gltA* and *gltB*, these sequences were conserved among different serotypes (Fig. 4 and 5). *Eco*RI restriction fragment length polymorphisms could be detected with probes derived from ORFZ and ORFP (Table 1). Sequences homologous to the ORFP- and ORFZ-derived probes were detected in other *Listeria* species as well, except for *L. grayi* and *L. welshimeri* (Table 1).

Serotype 1/2b *L. monocytogenes* harbors a novel locus genomically equivalent to the *gltA-gltB* cassette of serotype 4b-4d-4e. The genomic equivalent of the region flanked by the conserved ORFZ and ORFP was amplified from strain F4242 (serotype 1/2b) as described in Materials and Methods. In serotype 1/2b, ORFP and ORFZ flanked a region of 528 bp, in contrast to 3,071 bp in serotype 4b (Fig. 6). Interestingly, the region in serotype 1/2b was flanked by palindromic sequences with significant sequence identity (72 and 84%) to their counterparts in serotype 4b (Fig. 7). The remainder of the 528-bp region, however, showed no detectable homology with the serotype 4b sequences. The 1/2b sequence contained only a small potential coding sequence (ORFC, 75 amino acids), which was preceded by a putative Shine-Dalgarno sequence 7 nt upstream of the putative start codon. The palindromic sequence between ORFC and ORFP was followed by a string of eight T's, suggesting that it may function as a rho-independent terminator. The G+C content of ORFC was unusually low (26%), and no homologous sequences were identified in searches of the DNA and protein databases.

The sequenced 3′ portions of ORFP and ORFZ from serotype 1/2b were 97 and 98%, respectively, identical to their counterparts in serotype 4b. The corresponding C-terminal sequences of ORFP (144 residues) and ORFZ (31 residues) had 98 and 100%, respectively, identity, to their serotype 4b counterparts. Furthermore, Southern blots using probes derived from the regions outside the putative stem-loop structures (ORFP, ORFZ, and sequences distal to them) showed that the corresponding sequences and genomic organization were conserved between serotypes 4b and 1/2b (data not shown). The combined nucleotide sequence and Southern blot data suggest that the genomic organization of this region in serotypes 1/2b and 4b is as shown in Fig. 6.

TABLE 1. Southern blot hybridization data using probes from the *gltA-gltB* genomic region and *Eco*RI-digested DNAs from different strains of *Listeria*

Strain (serotype)	Hybridization with indicated DNA probe[a]			
	XL7-4 (ORFZ)	XL7-1 (*gltA*)	XL7-AB (*gltA-gltB*)	XL7-5 (ORFP-PRFO)
L. monocytogenes				
4b1 (4b)	5.0	5.0	5.0, 3.0	3.0
F2381 (4b)	4.5	4.5	4.5, 3.0	3.0
G2228 (1/2a)	2.6	0	0	6.0
F4242 (1/2b)	2.6	0	0	2.6
F4245 (1/2b)	2.6	0	0	5.0
LM103 (1/2c)	2.6	0	0	2.6
ATCC 19113 (3a)	2.6	0	0	2.6
G3331 (3b)	2.6	0	0	2.6
ATCC 2540 (3b)	3.0	0	0	2.6
G4315 (3c)	2.6	0	0	2.6
SLCC 2479 (3c)	2.6	0	0	2.6
ATCC 19114 (4a)	2.8	0	0	6.5
ATCC 19116 (4c)	6.5	0	0	6.5
ATCC 19117 (4d)	5.0	5.0	5.0, 3.0	3.0
ATCC 19118 (4e)	4.5	4.5	4.5, 3.2	3.2
G2940 (4ab)	7.5	0	0	1.5 (weak)
SLCC 2480 (7)	2.6	0	0	2.6
L. innocua				
120A1	4.2	0	0	1.5
F8596[b]	3.0	2.2	2.2, 1.7	1.5
G6882	4.2, 3.0	0	0	5.0, 1.5
G803	4.2, 3.0	0	0	5.0, 1.5
L. grayi	0	0	0	0
L. ivanovii	4.0	0	0	1.5
L. seeligeri	7.5 (weak)	0	0	7.5 (weak)
L. welshimeri	0	0	0	0

[a] DNA probes are as indicated in Materials and Methods and in Fig. 2 (thick lines). Values are the sizes of hybridizing fragments (in kilobases).
[b] Member of special lineage of *L. innocua* with serotype 4b-like TA composition (9).

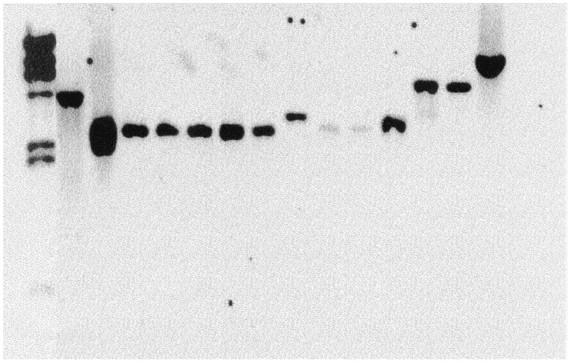

FIG. 4. Southern blot of *Eco*RI-digested genomic DNAs from *L. monocytogenes* of different serotypes, using the ORFZ-derived fragment XL7-4 (Fig. 1) as the probe. Lane 1, λ *Hin*dIII-digested molecular size markers (fragment sizes [from the top to bottom], 23, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kb); lanes 2 to 15, *L. monocytogenes* F2381 (4b), G2228 (1/2a), F4242 (1/2b), F4254 (1/2b), LM103 (1/2c), ATCC 19113 (3a), G3331 (3b), SLCC 2540 (3b), G4315 (3c), SLCC 2479 (3c), ATCC 19114 (4a), ATCC 19117(4d), ATCC 19118 (4e), and G2940 (4ab), respectively; lanes 16 and 17, *B. subtilis* 168 and *B. subtilis* W23, respectively. Lanes 10 and 11 contained relatively low amounts of DNA.

DISCUSSION

The ca. 3-kb gene cassette described here represents a novel serotype-specific locus present in serotype 4b *L. monocytogenes* and the genetically closely related (albeit relatively rare) serotypes 4d and 4e but in no other serotypes of the species. In addition, a unique *L. innocua* lineage that reacts with the serotype 4b-4d-4e-specific MAbs (9) also harbors the cassette, in the same genomic location as serotype 4b *L. monocytogenes*. The distribution of the cassette in *Listeria* parallels precisely the pattern of reactivity of the serotype-specific MAbs (8).

The genes on either side of the cassette were found to be conserved among different serotypes of *L. monocytogenes* as well as other *Listeria* species (*L. innocua, L. ivanovii*, and *L. seeligeri*). On one side, at least one of these genes (ORFP) may be involved in cell wall biosynthesis, the deduced product being a putative PBP. On the other side, we identified four genes with homology to ABC transporters. It remains to be determined whether the products of these genes mediate transport of cell wall or TA precursors.

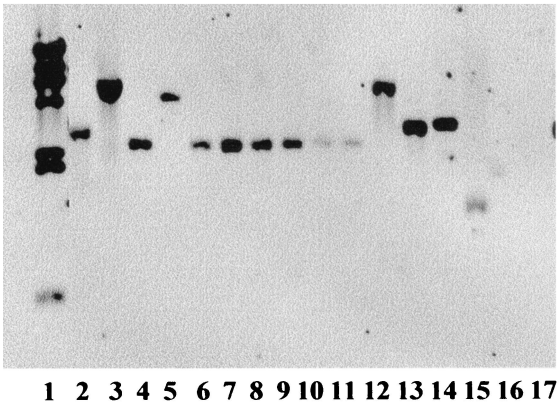

FIG. 5. Southern blot of *Eco*RI-digested genomic DNAs from *L. monocytogenes* of different serotypes, using the ORFP-ORFO-derived fragment XL7-5 (Fig. 1) as the probe. Lanes are identical to those in Fig. 4. The membrane used for the Southern blot in Fig. 4 was stripped of its probe and reprobed with XL7-5.

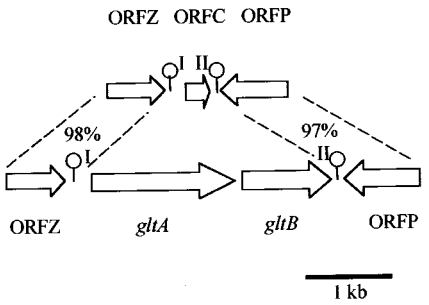

FIG. 6. Comparison between the *gltA-gltB* region of serotype 4b (bottom) and the genomically equivalent region in serotype 1/2b (top). Putative stem-loops I and II (lollipops) represent the boundaries of unique cassettes of different size (3,071 bp in serotype 4b and 528 bp in serotype 1/2b). The percentages indicate nucleotide sequence identities between the corresponding conserved ORFs on either side of the cassettes.

The serotype-specific distribution of the cassette and its unusually low (for *Listeria*) G+C content suggest the possibility that it may have been introduced to the *L. monocytogenes* serotype 4b-4d-4e lineage by horizontal transfer from some unidentified source. From there it could have been transferred to lineage I of *L. innocua*, as has been speculated for the *gtcA* locus recently identified in this lineage (9). The origin of the serotype-specific sequences may be elucidated by future identification of homologous sequences in other bacteria or bacteriophage. It is tempting to speculate that the inverted repeats flanking the serotype-specific sequences may represent remnants of a genetic system (e.g., a transposon or phage) that may have mediated this transfer. These palindromic sequences may have assumed novel functions in their current locations in serotype 4b *L. monocytogenes*, possibly related to transcriptional termination, message stability, or other regulatory mechanisms. Interestingly, these inverted repeats were similar to their counterparts in serotype 1/2b. In the latter, however, the genomic location of the ca. 3-kb serotype 4b-4d-4e-specific cassette was occupied by a much shorter (528-bp) region, which harbors a novel, unrelated ORF. Involvement of the 1/2b region (ORFC) in expression of surface antigen(s) in serotype 1/2b remains to be determined.

```
                    I
1/2b    122 AAAGTGAGTTCTTACGAGATTTTAGTAAGGACTCACTTT 160
            |||| |||| ||||     ||  ||||||| ||| ||||
4b     3350 AAAGCGAGTCCTTATCTTTTTCAAGTAAGGGCTCGCTTT 3388

                    II
1/2b    584 AAGAAGGTCGATTTCCTAATT.AGGAAATCGACCTTCTT 621
            || |  ||||||||||||||| ||||||||||  | ||
4b     6290 AAAAGAGTCGATTTCCTAATTCAGGAAATCGACTCTTTT 6328
```

FIG. 7. Comparison (BESTFIT) of the palindromic sequences corresponding to the putative stem-loops I and II in *L. monocytogenes* of serotypes 4b and 1/2b. Locations of putative stem-loops are as indicated in Fig. 6.

The integrated genetic, immunological, and biochemical results suggest that in *L. monocytogenes* serotype 4b, the *gltA-gltB* cassette is involved in expression of the surface antigens recognized by MAbs c74.22, c74.33, and c74.180 and in the addition of glucose substituents on the TA, but the precise biochemical functions of the two genes remain to be elucidated. The genes can be cotranscribed, and at this time we cannot exclude the possibility that the transposon insertion in *gltA* may have polar effects on *gltB*. The fact that the observed phenotypic complementation of XL7, albeit partial, was conferred equally by pKA and pKAB suggests that *gltB* was expressed to some extent in this mutant. Construction of alternative mutants in *gltA* (such as an in-frame deletion) and/or alternative complementation strategies will be needed to more precisely address the function(s) of *gltA*. Sequence analysis could not facilitate functional predictions in the case of *gltA*, as both the gene and the deduced gene product appeared to lack homologs in the databases. The deduced *gltB* product, however, had significant similarity with numerous glycosylases and dolichol phosphate mannosyltransferases, and a glycosylase function would be in agreement with the observed deficiency of glucose in the TA of the *gltB* mutant.

Complementation of MAb reactivity of XL7 by *gltA* or *gltA-gltB* was partial, for reasons that are not clear but may involve absence of possibly required *cis* elements or suboptimal copy number of the genes in the vector that was used. The low level of complementation may account for the lack of detectable restoration of glucose in the TAs. Such difficulties with complementation were not experienced with the previously studied gene *gtcA*, where both MAb reactivity and TA glycosylation were restored by the wild-type gene in *trans* (14). The mechanisms controlling regulation of expression of *gltA* and *gltB* are not understood but may be complicated, as suggested by the presence of the long and apparently transcribed region between ORFZ and *gltA*.

Glycosylated TA components have been shown to be important antigenic determinants in *L. monocytogenes* (6, 19), although their role in infection has not been elucidated. It is worthy of note that even though *gltA* or *gltB* mutants grew normally in the laboratory, our surveys of numerous serotype 4b field isolates (both food and clinical) failed to identify strains which had the XL7 or 4b1-INTB phenotype or which lacked *gltA-gltB* sequences. One may speculate that because of its surface exposure, abundance, and immunogenicity, properly decorated TA may be important in interactions between the bacteria and their host cells. Glycosylated TA may also affect physiological attributes of the bacteria in foods or in the environment, in response to environmental stresses, association with surfaces and with other organisms in biofilms, etc. Continuing studies in our laboratory aim toward further elucidation of the serotype-specific gene cassettes described in this report in terms of their evolution and potential roles in adaptive physiology and pathogenesis of the listerial lineages which harbor them.

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